Original Research Article

Identification and molecular characterization of bacteria and fungi associated with three fresh edible mushrooms

ABSTRACT

Need to describe the problem and the justification of the study.

Aims: To identify and characterize bacteria and fungi associated with three fresh edible mushrooms, under ambient and cold temperatures.

~~**Study design:** Experimental research design.~~

~~**Place and Duration of Study:** The Bells University of Technology, between December 2020 and October 2021.~~

Methodology: The study was conducted at the Bells University of Technology, Nigeria, between December 2020 and October 2021. *Pleurotus ostreatus* and *Calocybe indica* fruitbodies were procured from Federal Institute of Industrial Research Oshodi while *Pleurotus tuber-regium* fruitbodies were obtained from sclerotia planted in loamy soil. The fruitbodies were kept at ambient (28^{o}C) and cold (15^{o}C) temperatures, respectively. The bacterial and fungal counts on each of the mushrooms were taken at 0, 3, 5 and 7 days after harvest. The isolated bacteria were identified by conventional methods; Analytical Profile Index (API) 20E kits (BioMerieux), while Fungi were identified by morphological features and PCR amplification using ITS 1f/ ITS 4r universal primers.

Results: The bacterial and fungal counts on the fruitbodies ranged from 5.7 log cfu/ml – 6.3 log cfu/ml and 5.0 log cfu/ml – 5.9 log cfu/ml, respectively. Seven genera of bacteria isolated were gram negative bacteria. At ambient temperature, *Pseudomonas aeruginosa*, *Enterobacter asburiae*, *Klebsiella oxytoca*, *Klebsiella ornithinolytica*, *Serratia marcescens*, *Chryseobacterium meningosepticum* and *Cedecea davisae* were isolated while *Enterobacter cloacae*, *Enterobacter sakazakii* and *Citrobacter braakii* were isolated at cold temperature. *Aspergillus, Penicillium* and *Fusarium* were isolated at both temperatures while Alternaria was isolated only at ambient temperature.

Conclusion: Isolated bacteria and fungi were mostly enteric pathogens and potential mycotoxin producing fungi. This is an indication that strict hygiene and control measures should be put in place during production and storage of these mushrooms in order to improve the quality and food safety of the fresh mushrooms in Nigeria.

Keywords: *bacteria; fungi; identification; microbial counts; mushroom;storage temperature*

1. INTRODUCTION

Mushrooms are edible fungi with abundant nutritional and health benefits considered to be essential for humans [1,2]. They are rich in protein, vitamins, fiber, minerals, bioactive substances and are regarded as health food. In some parts of Nigeria, the locals use

mushrooms to replace meat in their diet such that mushrooms are gaining considerable attention and youths are being empowered to acquire skill in this regard.

Calocybe indica, Pleurotus ostreatus and *Pleurotus tuber-regium* are tropical edible mushrooms grown commercially in Nigeria and provide high returns for small scale farmers. Due to their nutrient content and high moisture content, the mushrooms are predisposed to different microorganisms and could harbour pathogenic microorganisms depending on cultural and environmental conditions of production and storage. Microbial infestation can occur during production, harvest, postharvest handling, storage, transportation, and processing. *Agaricus bisporus* mushrooms of good quality immediately after harvest developed brown blotches at retail, even while kept at refrigeration temperatures [3]. Temperature is one of the most measured factors accountable for mushroom deterioration and storage temperature is an important factor that affects postharvest life of mushrooms. Higher storage temperature can activate tyrosinase and increase microbial growth [1]. The study revealed that different bacteria and fungi were associated with the surface of fresh mushrooms at both ambient and refrigeration temperatures. Microbial growth is a function of variety of factors, both intrinsic (nutrient content, pH, water activity, physical structure of food oxidation-reduction potential) and extrinsic (temperature, relative humidity and food microbiota) [7]. Mushrooms are known to possess the above attributes, so fresh mushrooms can provide an ideal growth substrate for microorganisms. Authors need to modify references number in the text and ref. list…

Therefore, the identification of the microorganisms associated with fresh mushrooms is essential with respect to food safety, quality control, enhanced productivity and marketability. The study was to identify and characterize microorganisms associated with fresh *Calocybe indica, Pleurotus ostreatus* and *Pleurotus tuber-regium* under ambient and refrigeration temperatures.

2. MATERIALS AND METHODS

2.1 Materials

Calocybe indica and *Pleurotus ostreatus* were procured from Federal Institute of Industrial Research Oshodi, Lagos, Nigeria,–.while the sclerotia of *Pleurotus tuber-regium* purchased from a local market (Ojuore market in Ota, Ogun State), were planted in the soil, watered until fruitbodies were produced and harvested. The study was conducted during December 2020 and October 2021. The fruit bodies were kept at ambient (28^{o}C) and cold temperature (15^{o}C) respectively. Why the author(s) did not use the optimum refrigeration temperature of 10^{o}C? or may be lower…to compare it with other treatments.

2.2 Microbiological examination of mushroom samples

Bacterial and fungal counts were carried out, according to the method of [4]. The procedure was repeated on day 3, 5 and 7 respectively. Incubation of bacterial and fungal plates were at 37^{o}C and 28^{o}C, respectively.

2.3 Identification of bacterial isolates

The isolates were identified by conventional methods, morphological, cultural and Gram stain reactions. The Analytical Profile Index (API) 20E test kits by Bio Merieux SA was employed for biochemical identifications. The API kit was prepared according to the manufacturer's specification.

2.4 DNA extraction and PCR of fungal isolates

Fungi were identified using morphological and macroscopic features according to [5]. Extracted fungal DNA was amplified with ITS 1f/ ITS 4r universal primers for fungi according to [6].

3.0 RESULTS AND DISCUSSION

3.1 Total viable counts of bacteria isolate under ambient and cold temperatures

The study revealed that different bacteria and fungi were associated with the surface of fresh mushrooms at both ambient and refrigeration temperatures. Microbial growth is a function of variety of factors, both intrinsic (nutrient content, pH, water activity, physical structure of food oxidation-reduction potential) and extrinsic (temperature, relative humidity and food microbiota) [7]. Mushrooms are known to possess the above attributes, so fresh mushrooms can provide an ideal growth substrate for microorganisms Moved to the Introduction part…….

The total bacterial counts from the fresh mushrooms (day 0) at ambient temperature, were 5.8 log cfu/ml. The number increased up to the 7th day of storage except at day 5 for *C. indica* (Fig. 1). Continuous storage fruitbodies at ambient temperature, increased the bacterial counts up to 6.4 log cfu/ml, 6.2 log cfu/ml and 6.3 log cfu/ml over a 7-day period in *C. indica*, *P. ostreatus* and *P. tuber-regium*, respectively.Hope to estimate regression coefficient for the 3 mushroom varieties.

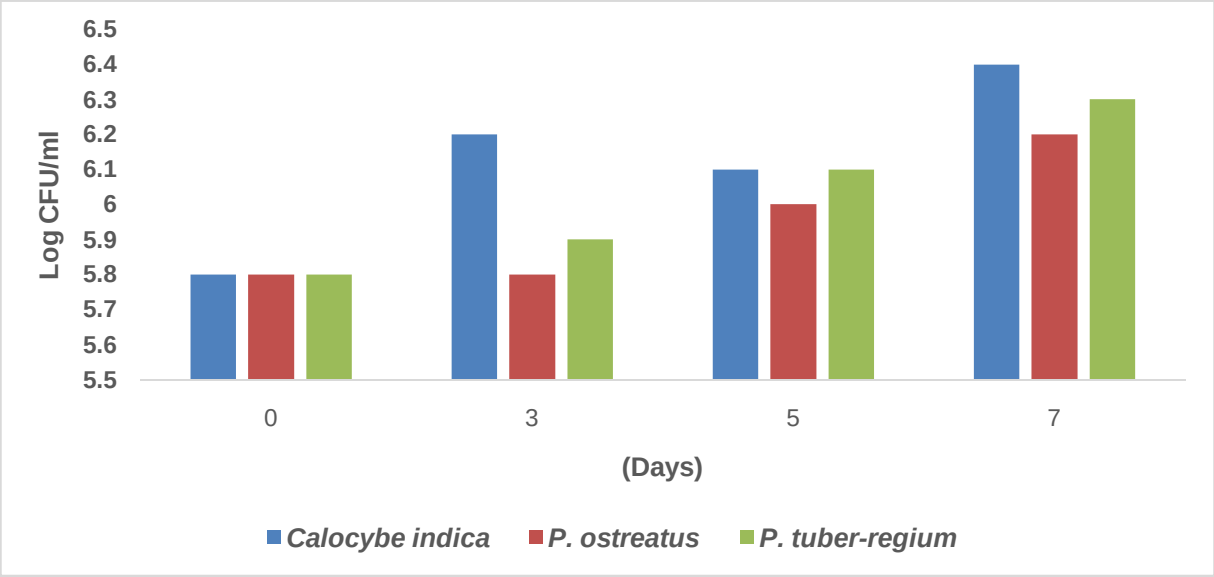


Fig. 1: Bacterial counts on fruitbodies of three fresh mushrooms stored under ambient temperature (28^{o}C)

This observation is in agreement with [3] who reported that in a 5-day post-harvest storage of sliced unwashed mushrooms, population of *Listeria monocytogenes* increased from 4 log cfu/g to 6.8 log cfu/g. Also, the brown blotch discoloration of *Agaricus bisporus* under refrigeration temperature was reported to be caused by high bacterial populations [8]. It was reported that 60% of fresh mushrooms and dried mushroom products examined in Bangladesh were contaminated with coliforms, *E coli*, *Salmonella* and the highest bacterial load recorded was 8.7 × 10^{8} cfu / gm [9]. Contrary to this observation, a low occurrence of pathogenic bacteria in fresh produce sampled in Norway was reported though the fresh mushrooms harboured non-toxinogenic *Staphylococcus aureus* [10]. Plate count of aerobic mesophilic microorganisms in food is one of the microbial indicators of food quality and large counts are regarded as harmful and reflect existence of favourable conditions for multiplication. The increasing number of aerobic counts showed that continued storage could be dangerous and may have serious consequences for food safety.

Under cold temperature, the increase in the bacterial counts were not so rapid until the 5th day (5.9 log cfu/ml - 6.1 log cfu/ml) and 7th day of storage (6.0 log cfu/ml - 6.3 log cfu/ml) (Figure 2). The result agrees with the suggestion of Singh *et al.,* [1] that higher storage temperature can activate tyrosinase and increase microbial growth. Based on this resut the authors should have been used lower refrigeration temperature than 15C. As well as not to have constant cold temperature???? I suggest to decrease cold temperature before the 5th day. Also to consider moisture during cold storage… This should be written in the recommendation of this study…. This was affirmed by [4] that temperature had significant effect on contaminants of some mushrooms. *Calocybe indica,* the milk mushroom supported the highest growth of microflora which could be due to the succulent nature and high nutrient status of the mushroom.

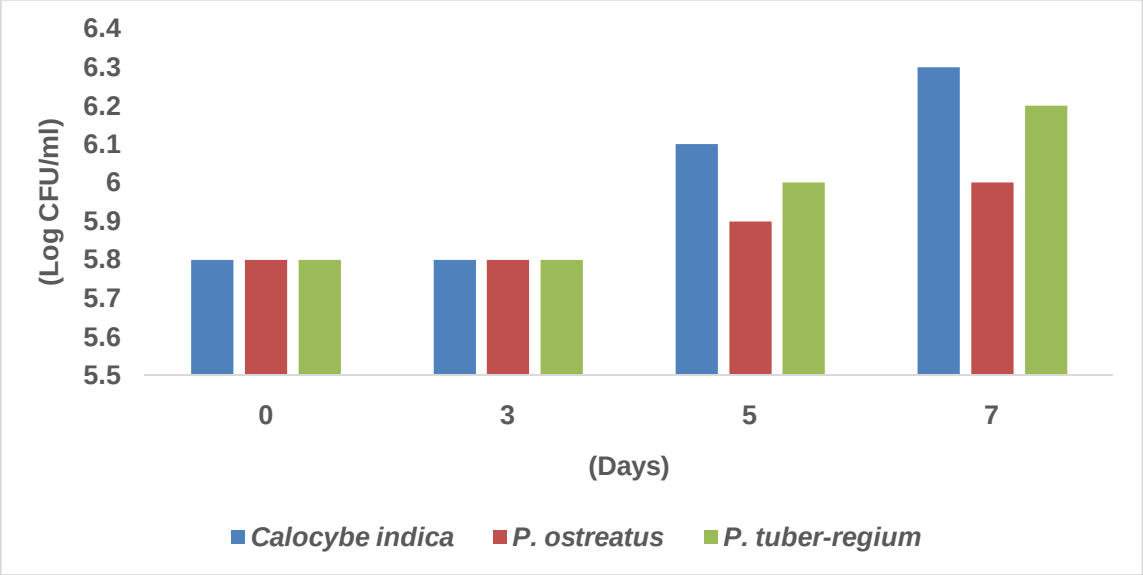


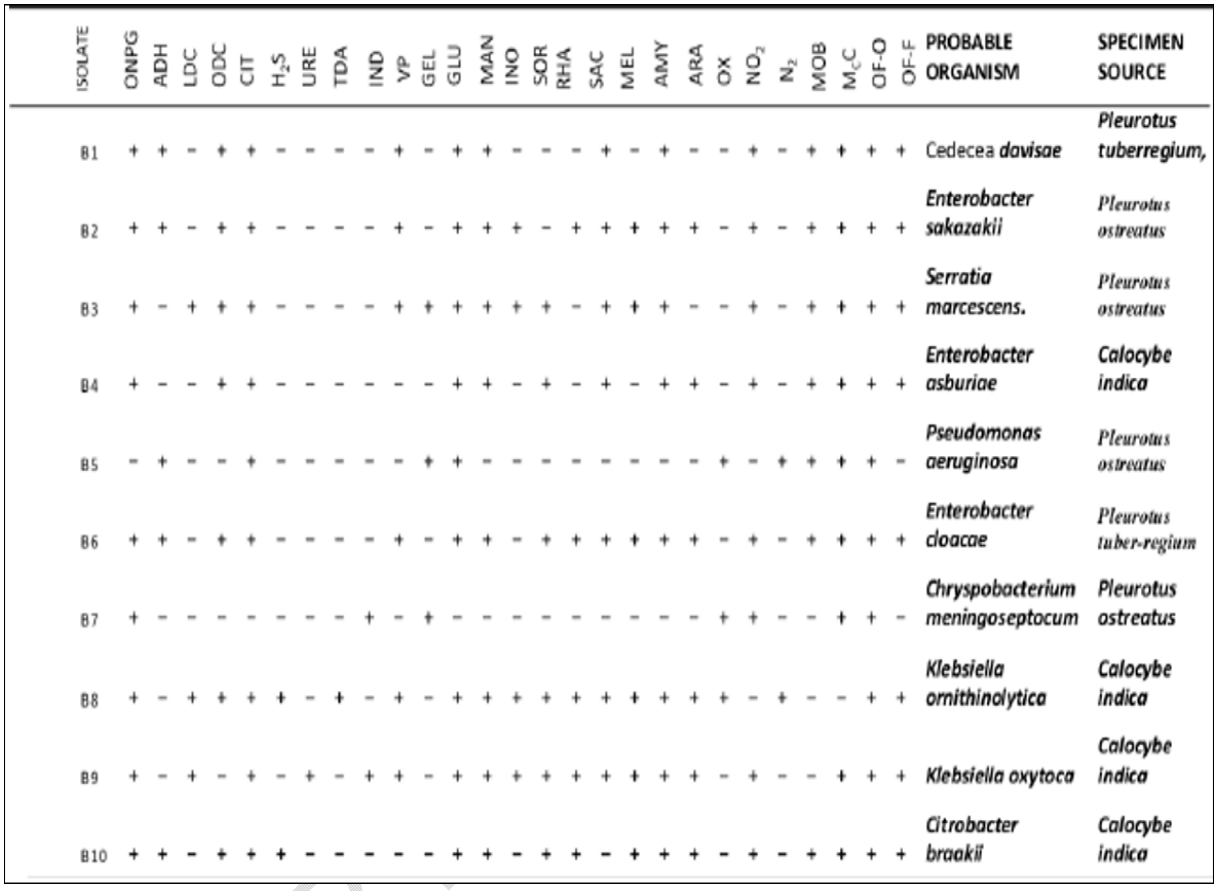
Fig. 2: Bacterial counts on fruitbodies of three fresh mushrooms stored under cold temperature (15°C)

Freshly harvested mushrooms were reported to harbour approximately 3 log cfu of moulds and 6 log cfu of native yeast per gram of fresh tissue and bacterial populations tend to increase from 7.3 to 8.4 log cfu g^{-1} during a 1-day storage period at $4^{o}C$ [11].

3.2 Identification of bacterial isolates using API 20E kits

Seven genera of bacteria belonging to the family Enterobacteriaceae were identified by the Analytical Profile Index 20E Kits; among them were *Enterobacter*, *Pseudomonas*, *Chryseobacterium*, *Klebsiella, Citrobacter*, *Serratia* and *Cedecea species.* Dominant among them were *Enterobacter* (30%) and *Klebsiella* species (20%). In another study, *Enterobacter* specie was among the eight bacteria isolated from diseased mycelia of *Pleurotus eryngii* [12]. In a similar study, *Enterobacter* and *Pseudomonas* were associated with the substrate used in the cultivation of *P. ostreatus* [13]. Most Enterobacteriaceae are mesophiles and thrive better under ambient temperature than refrigeration temperature [14]. This was corroborated by [7] that Enterobacteriaceae are slow growers under chill temperature and become more significant as temperature rises. Involvement of members of Enterobacteriaceae is indicative of environmental contamination as members of this family are enteric organisms spread through unhygienic handling of foods. These organisms are of public health concern as they are capable of causing serious ill health. Their presence in food could have serious implication for food safety and is indicative of poor hygiene. This calls for adequate hygiene and good manufacturing practice during production and storage. Mushrooms should be produced and processed observing good hygienic practices (GHP) and consumed soon after harvest. Please give some details for such hygienic practice…in the Introduction part….why authors did not use such practice as experimental treatments???…..I am surprising why such several genera of bacteria were found ??? Does using hot air for few seconds before cold storage may decrease bacteria count???

Image1:



ISOLATE	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO_2	N_2	MOB	M_2C	OF-O	OF-F	PROBABLE ORGANISM	SPECIMEN SOURCE
B1	+	+	-	+	+	-	-	-	-	+	-	+	+	-	-	-	+	-	+	-	-	+	-	+	+	+	+	Cedecea *davisae*	*Pleurotus tuberregium,*
B2	+	+	-	+	+	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+	*Enterobacter sakazakii*	*Pleurotus ostreatus*
B3	+	-	+	+	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	+	-	+	+	+	+	*Serratia marcescens.*	*Pleurotus ostreatus*
B4	+	-	-	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	+	+	+	+	*Enterobacter asburiae*	*Calocybe indica*
B5	-	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	*Pseudomonas aeruginosa*	*Pleurotus ostreatus*
B6	+	+	-	+	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+	*Enterobacter cloacae*	*Pleurotus tuber-regium*
B7	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	*Chryspobacterium meningoseptocum*	*Pleurotus ostreatus*
B8	+	-	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	*Klebsiella ornithinolytica*	*Calocybe indica*
B9	+	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	*Klebsiella oxytoca*	*Calocybe indica*
B10	+	+	-	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	+	+	-	+	-	+	+	+	+	*Citrobacter braakii*	*Calocybe indica*

Key: ONPG- Ortho-Nitrophenyl-β-galactoside, ADH- Arginine DiHydrolase, LDC- Lysine Decarboxy, ODC- Ornithine DeCarboxylase, CIT- Citrate, H2S- Hydrogen Sulphide Production, URE- Urease, TDA- Tryptophane DeAminase, IND- Indole Production, VP-Voges Praskauer, GEL- Gelatinase, GLU- D-Glucose, MAN- D-Mannitol, INO- Inositol, SOR- D-Sorbitol, RHA- L-Rhannose, SAC- Saccharose (D-Sucrose), MEL- D-Melibiose,

This is contrary to previous reports that members of *Pseudomonads* were the dominant species [11]; the occurrence of *Pseudomonas* in this work was only 10% and is similar to

[15] who reported 8% on vegetables. Bacterial species isolated at ambient temperature were *Pseudomonas aeruginosa*, *Serratia marcescens* and *Chryseobacterium meningosepticum* from *P. ostreatus*; *Cedecea davisae* from *P. tuber-regium* while *Enterobacter asburiae, Klebsiella ornithinolytica* and *Klebsiella oxytoca* were isolated from *Calocybe indica*. At cold temperature, *Enterobacter sakazaki, Enterobacter cloacae* and *Citrobacter braakii* were isolated from *P. ostreatus, P. tuber-regium* and *C. indica*,, respectively. Any reason?? Please discus.

3.3 Total viable counts of fungal isolates under ambient and cold temperatures

The fungal counts under ambient temperature ranged from 5.0 log cfu/ml to 5.9 log cfu/ml (Fig. 3) while at cold temperature the range was 5 log cfu/ml to 5.6 log cfu/ml (Fig. 4). Same trend was observed for fungal counts as the counts at ambient temperature exceeded that of cold storage. Bacterial counts were more than fungal counts and increased with storage. This could be due the rapid generation time of bacteria compared to fungi. Any reason why C. indica showed higher count of fungal isolates under cold temperature than other mushroom???

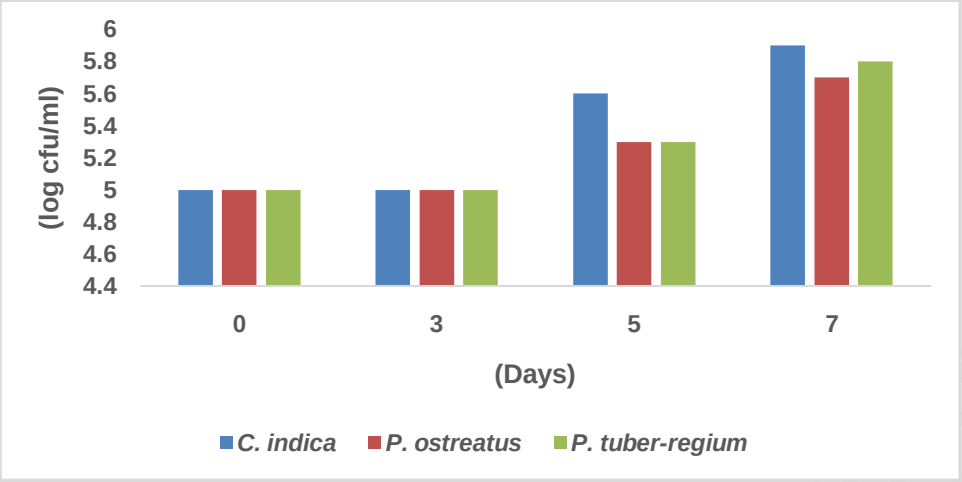


Fig. 3: Total viable counts of fungal isolates at ambient temperature

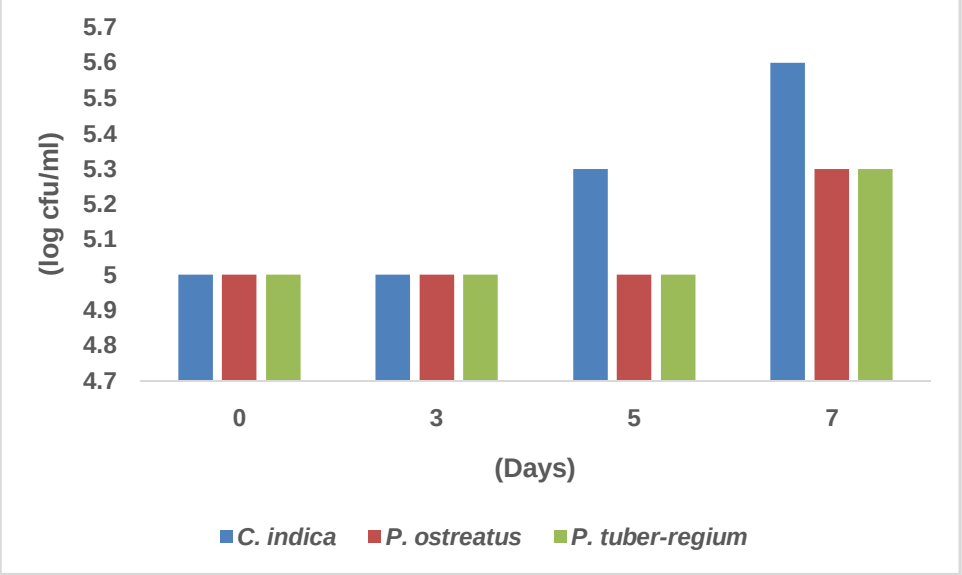


Fig 4: Total counts of fungal isolates under cold temperature

3.4 Identification of fungal isolates by colony morphology

Four genera of fungi were isolated from the mushrooms, three fungal isolates were isolated under both ambient and cold temperatures and include *Aspergillus spp., Penicillium spp.,* and *Fusarium spp.* While *Alternaria spp.* was found under ambient condition (Table 1). The dominant fungal species were *Aspergillus* (41%) and *Penicillium (*25%). This observation was corroborated by[13] as *Aspergillus* had the highest frequency of occurrence.

Table 1: Colony Morphology of isolated fungi from *C. indica*, *P. tuber-regium* and *P. ostreatus*

Mushroom species	Probable Organism	Colour	Conidio-phore	Phalides	Vesicle	A/R	Isolates
P. ostreatus	*Alternaria sp.*	Dark-brown	Septate	uniseriate	Zigzag	A	F1
P. ostreatus	*Aspergillus sp.*	Yellow-green	glubose	uniseriate	round	A	F2
P. ostreatus	*Penicillium sp.*	Greenish	downy	uniseriate	round	R	F8
P. ostreatus	*Aspergillus sp.*	Blue-green	smooth	uniseriate	round	R	F12
C. indica	*Penicillium sp.*	Greenish	smooth	uniseriate	round	A	F3
C. indica	*Fusarium sp.*	Whitish	smooth	uniseriate	round	A	F4
C. indica	*Fusarium sp.*	Whitish	smooth	uniseriate	round	R	F5
C. indica	*Aspergillus sp.*	yellow-green	round	uniseriate	round	A	F6
C. indica	*Aspergillus sp.*	black	smooth	biseriate	round	R	F7
C. indica	*Aspergillus sp.*	blue-green	septate	uniseriate	round	A	F13
P. tuber-regium	*Aspergillus sp.*	Blue-green	smooth	uniseriate	round	A	F9
P. tuber-regium	*Aspergillus sp*.	Yellow-green	smooth	uniseriate	round	A	F10
P. tuber-regium	*Penicillium sp.*	Greenish	smooth	uniseriate	round	R	F11

A/R means ambient /refrigeration.

Aspergillus, Fusarium and *Penicillium* were among the fungi isolated from the substrates used in cultivation of *P ostreatus* [13].

3.4 Molecular identification of fungal isolates under ambient and cold temperatures

Molecular approaches for identification of fungi included internal transcribed spacer region (ITS) and had been used for detection of fungi. The PCR fragment sizes of fungal isolates using ITS1f and ITS4r were observed at about 600 bp for all isolates (fig. 5).

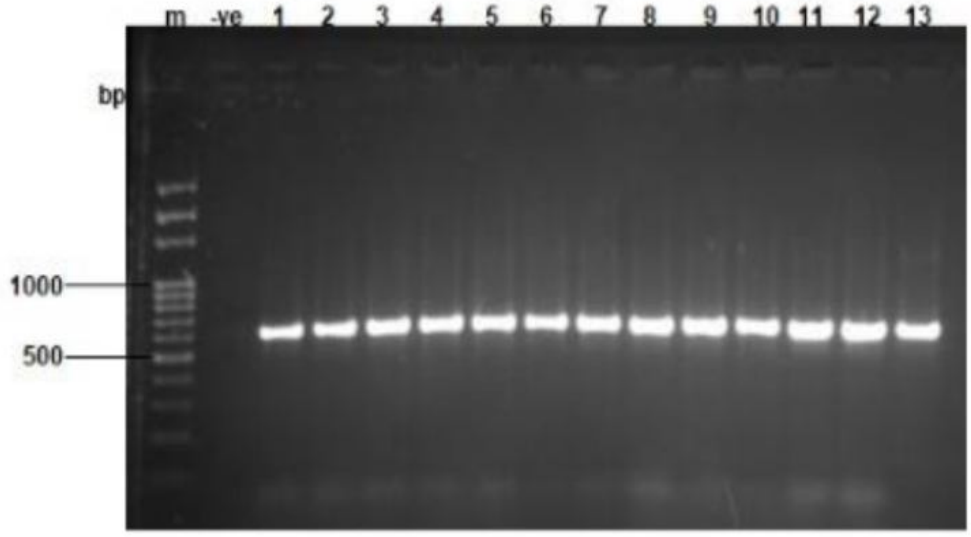


Fig. 5: PCR Amplification of fungal isolates using ITS1F/ITS4R Universal Primers and 100 bp ladder.
m-molecular ladder; -ve- negative control; 1-13- fungal isolates

Similar fragment size was obtained by [6] for isolates of *Aspergillus* species.

Storage temperature seems to have an effect on microbial counts as more numbers were observed under ambient temperature.

The implications of these results are that mushrooms could harbour large numbers of pathogenic organisms during post-harvest and storage periods.

4. CONCLUSION

Many enteric bacteria and potential mycotoxin producing fungi were associated with the three edible mushrooms both at ambient and cold temperatures. The bacteria identified include *Enterobacter asburiae, Klebsiella oxytoca, Klebsiella ornithinolytica, Pseudomonas aeruginosa, Serratia marcescens, Chryseobacterium meningosepticum, Cedecea davisae, and Citrobacter braakii* under ambient while under cold storage, *Enterobacter cloacae, Enterobacter sakazakii* and *Citrobacter braakii* were identified. The fungal species include *Aspergillus, Penicillium, and Fusarium* under both temperatures. Only *Alternaria spp.* was isolated under ambient temperature. The implication is that strict hygiene should be maintained during mushroom cultivation and post-harvest operations.

With such hard work, what is your practical recommendations to get better healthy food?

CONSENT (WHERE EVER APPLICABLE)

This is not applicable

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

THIS IS NOT APPLICABLE

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